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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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THIS IS A REQUEST FOR FILING A PROVISIONAL APPLICATION FOR PATENT UNDER 37 C.F.R. § 1.53(c)

60/277947 U.S. PTO 03/23/01

INVENTOR(S)/APPLICANT(S)

Given Name (first and middle (if any))	Family Name or Surname	Residence (City and Either State or Foreign Country)
David J.	KYLE	Catonsville, Maryland

☐ Additional inventors are being named on page 2 attached hereto.

TITLE OF THE INVENTION (280 characters max)

DELIVERY OF DISEASE CONTROL IN AQUACULTURE AND AGRICULTURE USING MICROBES CONTAINING BIOACTIVE PROTEINS

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification Number of Pages 8

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☒ Small Entity Status Claimed As:
- ☒ Independent Inventor
- ☐ Small Business Concern
- ☐ Nonprofit Organization
- ☐ Non-Inventor Supporting Claim By Another

☐ Other (specify) _____

METHOD OF PAYMENT OF FILING FEE FOR THIS PROVISIONAL APPLICATION

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☐ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

By Laurence H. Posorske

Laurence H. Posorske

Date March 23, 2001
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Delivery of Disease Control in Aquaculture and Agriculture Using Microbes Containing Bioactive Proteins.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention is directed to the use of microbial cells which are used as feed components in aquaculture or agriculture, and which also contain exogenous peptides and/or antibodies which will convey resistance or immunity to viral or bacterial pathogens or otherwise improve the health and performance of the species consuming said microbial cells. The microbial cells can be yeast, fungi, bacteria, or algae. The proteins and/or antibodies may be expressed inside the microbial cells by direct genetic modification of the microbe itself, or by the infection of the microbe with a virus that has been altered to express the protein of interest.

Related Art

Certain plant products have been produced using specific genetic modification to express proteins and/or antibodies of therapeutic value. The group at the Boyce Thompson Institute at Cornell has been cloning viral coat protein into bananas so that when ingested, this will be equivalent to delivering an oral vaccine. This concept has not been extended to microbes.

There are several plant biotech companies such as Meristem, Large Scale Biology, and Prodigene, which are now expressing certain human therapeutic proteins in the plants including antibodies.

Recombinant microbes including bacteria, yeast and fungi have been used to produce human therapeutic proteins. However, such recombinant microbes have not been used for agricultural purposes incorporating ingestion of the whole organism. In both the plant and microbial cases, the recombinant organism has simply been used as a factory, and the therapeutic protein is then isolated and purified prior to use.

Certain plant products have been produced which contain proteins and/or antibodies of therapeutic value by infecting the plant with a virus that expresses the protein of interest.

Large Scale Biology has a series of patents protecting this technology but these patents do not disclose the use of the technology in microbes and certainly not algae.

Antibiotic doping is used routinely in the aquaculture setting. Typically, the pure or semipure antibiotics are added directly to the water column; however, crude fermentation broths, particularly broths including cells, have not been used for any kind of therapeutic delivery system.

Production of amino acids such as lysine typically involves a genetically modified microorganism which overproduces the amino acid of interest and excretes it into the fermentation medium. The wastestream from such a fermentation would include biomass containing the amino acid, and this wastestream product could be used as a crude delivery form of the small molecule nutritive amino acid.

SUMMARY OF THE INVENTION

The present invention provides for a composition of matter (the feed) and the use of this feed for the delivery of a therapeutic dose of a bioactive peptide or protein.

In one embodiment, this invention provides an aquaculture feed containing microbial biomass comprising one or more proteins, antibodies, or a combination thereof, where the proteins and antibodies are non-native to the microbes of the biomass. Preferably, the microbes are selected from yeast, fungi, bacteria, algae, or combinations thereof. The microbes may express the proteins or antibodies recombinantly, or the microbes may be infected with viruses which express the proteins or antibodies recombinantly.

In another embodiment, this invention provides a method of delivering therapeutic proteins to a non-human animal comprising administering to a non-human animal a feed comprising a microbe expressing a non-native therapeutic protein. This method is particularly suitable for the non-human animal subjected to intensive agricultural practices, or for fish or shellfish in aquaculture. Preferred microbes are algae. In a preferred mode, the therapeutic protein is a recombinant protein expressed by the microbe or the microbe is infected by a recombinant virus which expresses the therapeutic protein recombinantly. Preferred therapeutic proteins include a protein which inhibits growth or replication of Vibrio species in vitro, or a protein which inhibits Taura or White spot virus infection in shrimp, or a recombinantly expressed antibody.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Microalgae (single cell algae, or phytoplankton) represent the largest, but most poorly understood, kingdom of microorganisms on the earth. Like plants are to terrestrial animals, the microalgae represent the natural nutritional base and primary source of all the phytonutrients in the aquatic food chain. As the primary link in the aquatic food chain, microalgae are the source of many more phytonutrients than simply DHA and ARA. Microalgae also represent a vast genetic resource comprising in excess of 80,000 different species. Yeast, fungi and bacterial are also in the direct food chain of fish, crustaceans and mollusks. However, only a very few of these microbes, perhaps less than 10 species, have been exploited for aquaculture feeds. These few species have been used primarily for historical reasons and ease of cultivation. They have not been chosen on the basis of any scientific evidence of superiority as nutritional or therapeutic supplements.

The marine environment is filled with bacteria and viruses that can attack fish and shellfish and devastate intensive farms very quickly. Bacteria and viruses can also attack single cell microalgae so these organisms have evolved biochemical mechanisms to defend themselves from such attacks. Such mechanisms may involve the secretion of compounds that inhibit bacterial growth or viral attachment. Such compounds are called "prebiotics" and have effects similar to how cranberry juice can prevent bladder infections in humans. When delivered as the whole live organisms, such as *Lactobacillis* in yoghurt, they are referred to as "probiotics".

The inventor has undertaken a screening program and found that, unexpectedly, several algal species exhibit antibiotic activity. This activity may be due to certain bioactive constituents in the membranes or cell walls, the protein or the carbohydrate of the positively testing species that inhibit bacterial growth (prebiotics). Any standard screening technique used to identify antibiotic agents may be used to screen for algae having antibiotic activity, including incubating filter disks soaked in culture broth from the candidate algae on a lawn of the target pathogenic microbe (e.g., *Vibrio* species). This invention contemplates the use of these "friendly algae" in a probiotic fashion to control the growth of certain "pathogenic microorganisms" in a pond. However, the main aspect of this invention is directed to the use of recombinant microbes or virus infected microbes to deliver the bioactive protein of choice.

The recombinant microbes or virus-infected microbes may be tested for antibiotic activity by standard antibiotic screening assays to confirm their activity.

Historically, only bacteria have been used in a probiotic fashion to alter a pond's ecology in order to eliminate or reduce the number of pathogenic bacteria. A problem with the bacterial probiotics approach is that the existing microbial ecology represents a massive buffer that is difficult to modulate with the introduction of relatively small numbers of alternative bacteria and the results to date have been unimpressive. Furthermore, even if the newly introduced bacteria do bloom, any large increase in bacterial levels in a pond can lower oxygen levels and cause harm the fish or shrimp. The use of photosynthetic microalgae overcomes this problem as they actually increase oxygen levels. Microalgae have not been considered before as probiotics because bacteria-controlling species of algae have, heretofore, not been discovered. Previous experience in the screening of an extensive algal culture collection has indicated a number of algal species that exhibit antibacterial or bacteriostatic capabilities. Some of these activities may be anti-Vibrio activity. Such species would be candidates for a high value enrichment feed that delivers both nutritional and antibiotic capabilities. This invention provides an approach to disease control which may be the solution to an impending ecological disaster that will result from the present uncontrolled practice of dumping of toxic chemicals and antibiotics into the water systems to control these pathogens.

One of the major disease control problems in shrimp aquaculture today is infection by certain viruses (e.g., White Spot). Neither antibiotic, nor probiotic strategies will work in this situation, and shrimp cannot be vaccinated in a way similar to fish. Shrimp have only a rudimentary immune system so they are particularly susceptible to devastation by viral attacks. This invention provides a solution to this problem using a biological control method using microalgae as the vector to deliver anti-White Spot antibodies directly to the shrimp. These "Designer feeds" would be a normal part of the diet, but modified to deliver a therapeutic dose of antibody directly to the gut of the shrimp. This approach is known as "passive immunity" because the antibody remains outside the host organism and simply prevents infestation through the gut wall. The invention envisions the use of trangenic algae, yeast, fungi or bacteria to deliver the antibody to the virus. Alternatively, the microbe itself may be infected with a virus that is engineered to produce the antibody of interest. Alternatively, the microbial source may deliver a portion of the virus (e.g. a coat protein) or fragment thereof in order to immunize the shrimp, other shellfish or finfish.

Antibodies to desired targets, such as White Spot virus or Taura virus, may be prepared by routine immunization and selection of monoclonal antibody producing hybridomas, or by screening viral or bacterial expression libraries of immunoglobulin genes and gene fragments. See "Current Protocols in Immunology," Coligan, et al., eds, Wiley-Interscience, 1991, and periodic supplements. Nucleic acid sequences encoding the binding sites of the selected antibodies can be cloned using standard methods (see "Current Protocols in Molecular Biology." Ausubel, et al., eds, Wiley-Interscience, 1987, and periodic supplements), and antibodies may be expressed from recombinant microbes (including algae, see, e.g., U.S. Patent No. 6,027,900) or cloned into viruses that infect the desired microbes.

There are a number of well known bactericidal and bacteriostatic peptides which will inhibit microbial growth and include, but are not limited to cecropins, peneadins, bactenecins, callinectins, myticins, tachyplesins, clavanins, misgurins, pleurocidins, parasins, histones, acidic proteins, and lysozymes. These peptides may be made in a microbial biomass such as algae, yeast, fungi or bacteria using recombinant methods as described above, and thus provided as a feed component to convey resistance to infestation.

EXAMPLES

The invention as contemplated herein, is described in the following examples, but its utility is not limited to the examples provided.

Example 1. Selection of Useful Microbial Sources for Feeds that Provide Disease Control. Microalgal biomass samples, aqueous extracts, organic extracts and extracts from the growth medium after cultivation of the algae were concentrated and spotted on filter paper discs. Using sterile techniques, these discs were then placed on agar plates overlaid with a lawn of selected organisms including but not limited to gram negative bacteria, gram positive bacteria, antibiotic resistant bacteria, yeast, or fungi. After incubation for an appropriate length of time to allow growth of the lawn of test organism the samples were plates were observed for zones of clearing (non-growth) around the filter paper discs. Large zones of clearing indicate potent antibiotic activity, small zones of clearing indicate less potent antibiotic activity. Following such a test, numerous algae were found to have moderate to good antibiotic activity:

Example 2. Incorporation of an antibody into an algal feed. A particular viral or bacterial pathogen is chosen and used to prepare monoclonal antibodies using procedures well known to experts in this field. Gene(s) coding for this antibody or an appropriate antibody fragment (FAB) are isolated and amplified in the appropriate vector. The gene is spliced into a transformation vector suitable for a eukariotic algae (e.g. *Chlorella*) or a prokariotic alga (eg. *Synechocystis*), or a yeast (e.g. *Saccharomyces*) or a fungi (eg. *Mortierella*). The transformation vector is chosen so that the antibody will be overexpressed in the microbial cell biomass. This biomass is then used as a feed additive in such a way as to provide the antibody directly to the animal thus providing passive immunity.

Example 3. Expression of a bactericidal protein in a microbial feed. A bactericidal protein is chosen for the particular application. For example, proteins of the penaeidin class may be chosen for pathogenic control in shrimp. Penaeidins are members of a family of antimicrobial peptides isolated from crustaceans (eg., Penaeus shrimp). Antimicrobial peptides may also come from insects and chelicerates and may include but are not limited to cecropins, peneadins, bactenecins, callinectins, myticins, tachyplesins, clavanins, misgurins, pleurocidins, parasins, histones, acidic proteins, and lysozymes. The gene for the chosen protein or peptide is either isolated from the original source, an amplification source, or it can be made synthetically. The gene is then incorporated into a transformation vector suitable for a eukariotic algae (e.g. *Chlorella*) or a prokariotic alga (eg. *Synechocystis*), or a yeast (e.g. *Saccharomyces*) or a fungi (eg. *Mortierella*). The transformation vector is chosen so that the protein will be overexpressed in the microbial cell biomass. This biomass is then used as a feed additive in such a way as to provide the bactericidal protein directly to the animal thus providing resistance to that particular pathogen.

Example 4. Vaccination using Feeds. An antigen characteristic to a particular pathogen is chosen as is required by the animal and circumstances. For example, a viral coat protein or component thereof, or a infectious bacterial protein, or a component thereof is chosen. The gene coding for the protein is isolated and incorporated into a vector suitable for use in the microorganism of choice. The transformation vector is chosen so that the protein will be overexpressed in the microbial cell biomass. This biomass is then used as a feed additive in

such a way as to provide the viral or bacterial or fungal protein directly to the animal thus stimulating an immunological response to that particular pathogen. The microbial component may enter the body of the animal in the digestive tract, or otherwise through contact in the air or water.

CLAIMS

An aquaculture feed containing microbial biomass comprising one or more proteins, antibodies, or a combination thereof, wherein said proteins and antibodies are non-native to the microbes of the biomass.

The aquaculture feed wherein the microbes are selected from yeast, fungi, bacteria, algae, or combinations thereof.

The aquaculture feed wherein the microbes express the proteins or antibodies recombinantly.

The aquaculture feed wherein the microbes are infected with viruses which express the proteins or antibodies recombinantly.

A method of delivering therapeutic proteins to a non-human animal comprising administering to a non-human animal a feed comprising a microbe expressing a non-native therapeutic protein. .

The method of delivering therapeutic proteins wherein the non-human animal is subjected to intensive agricultural practices.

The method of delivering therapeutic proteins wherein the non-human animal is fish or shellfish in aquaculture.

The method of delivering therapeutic proteins wherein the microbe is an alga.

The method of delivering therapeutic proteins wherein the therapeutic protein is a recombinant protein expressed by the microbe.

The method of delivering therapeutic proteins wherein the microbe is infected by a recombinant virus which expresses the therapeutic protein recombinantly.

The method of delivering a therapeutic protein wherein the therapeutic protein is a protein which inhibits growth or replication of *Vibrio* species *in vitro*.

The method of delivering a therapeutic protein wherein the therapeutic protein is a protein which inhibits Taura or White spot virus infection in shrimp.

The method of delivering a therapeutic protein wherein the therapeutic protein is a recombinantly expressed antibody.

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27(b)) - INDEPENDENT INVENTOR	Docket No. Kyle-1

Application No.	Filing Date	Patent No.	Issue Date
To Be Assigned	March 23, 2001		

Applicant/ Patentee: David J. Kyle

Invention: DELIVERY OF DISEASE CONTROL IN AQUACULTURE AND AGRICULTURE USING MICROBES CONTAINING BIOACTIVE PROTEINS

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled above and described in:

- [X] the specification to be filed herewith.
- [ ] the application identified above.
- [ ] the patent identified above.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- [X] No such person, concern or organization exists.
- [ ] Each such person, concern or organization is listed below.

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME _____

ADDRESS _____

[ ] Individual [ ] Small Business Concern [ ] Nonprofit Organization

FULL NAME _____

ADDRESS _____

[ ] Individual [ ] Small Business Concern [ ] Nonprofit Organization

FULL NAME _____

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF INVENTOR David J. KYLE

SIGNATURE OF INVENTOR _____ DATE: 03/23/01

NAME OF INVENTOR _____

SIGNATURE OF INVENTOR _____ DATE: _____

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WO 02/076391 A2

(54) Title: DELIVERY OF DISEASE CONTROL IN AQUACULTURE AND AGRICULTURE USING MICROBES CONTAINING BIOACTIVE PROTEINS

(57) Abstract: An animal feed contains microbial biomass comprising one or more proteins, antibodies, therapeutics, or a combination thereof, wherein said proteins and antibodies are non-native to the microbes of the biomass. The proteins can be therapeutic, bioactive, or nutritional proteins. A vaccine can be employed in bacteria to immunize an animal. Alternatively, an antimicrobial compound can be employed in bacteria.

Delivery Of Disease Control In Aquaculture and Agriculture Using Microbes Containing Bioactive Proteins

BACKGROUND OF THE INVENTION

Field of the Invention

[001] This invention is directed to the use of microbial cells which are used as feed components in aquaculture or agriculture, and which also contain exogenous peptides, proteins, and/or antibodies, which will convey resistance or immunity to viral or bacterial pathogens or otherwise improve the health and performance of the species consuming said microbial cells. The microbial cells can be yeast, fungi, bacteria, or algae. The proteins and/or antibodies may be expressed inside the microbial cells by direct genetic modification of the microbe itself, or by the infection of the microbe with a virus that has been altered to express the protein of interest.

Related Art

[002] Certain plant products have been produced using specific genetic modification to express proteins and/or antibodies of therapeutic value. The group at the Boyce Thompson Institute at Cornell has been cloning viral coat protein into bananas and potatoes so that when ingested, this will be equivalent to delivering an oral vaccine. This concept has not been extended to microbes.

[003] There are several plant biotech companies such as Meristem, Large Scale Biology, and Prodigene, which are now expressing certain human therapeutic proteins in the plants including antibodies.

[004] Recombinant microbes including bacteria, yeast and fungi have been used to produce human therapeutic proteins. However, such recombinant microbes have not been used for agricultural purposes incorporating ingestion of the whole organism. In both the plant and microbial cases, the recombinant organism has simply been used as a factory, and the therapeutic protein is then isolated and purified prior to use.

[005] Certain plant products have been produced which contain proteins and/or antibodies of therapeutic value by infecting the plant with a virus that expresses the protein of interest. Large Scale Biology has a series of patents protecting this technology but these patents do not disclose the use of the technology in microbes and certainly not algae.

[006] Antibiotic doping is used routinely in the aquaculture setting. Typically, the pure or semipure antibiotics are added directly to the water column; however, crude fermentation broths, or crude preparations including cells, have not been used for any kind of therapeutic delivery system.

[007] Production of amino acids such as lysine typically involves a genetically modified microorganism, which overproduces the amino acid of interest and excretes it into the fermentation medium. The wastestream from such a fermentation would include biomass containing the amino acid, and this wastestream product could be used as a crude delivery form of the small molecule nutritive amino acid.

SUMMARY OF THE INVENTION

[008] The present invention provides for a composition of matter (the feed) and the use of this feed for the delivery of a therapeutic dose of a bioactive peptide or protein.

[009] In one embodiment, this invention provides an aquaculture feed containing microbial biomass comprising one or more proteins, antibodies, or a combination thereof, where the proteins and antibodies are non-native to the microbes of the biomass. Preferably, the microbes are selected from yeast, fungi, bacteria, algae, or combinations thereof. The microbes may be engineered to recombinantly express the proteins or antibodies recombinantly, or the microbes may be infected with viruses or plasmids, which express the recombinant proteins or antibodies.

[010] In another embodiment, this invention provides a method of delivering therapeutic proteins to an animal comprising administering to an animal a feed comprising a microbe expressing a non-native therapeutic protein. This method is particularly suitable for the non-human animal subjected to intensive agricultural practices, or for fish or shellfish in aquaculture. Preferred microbes are algae. In a preferred mode, the therapeutic protein is a recombinant protein expressed by the microbe or the microbe is infected by a recombinant virus, which expresses the recombinant therapeutic or bioactive protein. Preferred therapeutic proteins include a protein which inhibits growth or replication of *Vibrio* species in vitro, or a

protein which inhibits Taura Syndrome Virus (TSV) or White Spot Syndrome Virus (WSSV) infection in shrimp, or a recombinantly expressed antibody.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[011] Microalgae (single cell alga, or phytoplankton) represent the largest, but most poorly understood, kingdom of microorganisms on the earth. Like plants are to terrestrial animals, the microalgae represent the natural nutritional base and primary source of all the phytonutrients in the aquatic food chain. As the primary link in the aquatic food chain, microalgae are the source of many more phytonutrients than simply DHA and ARA. Microalgae also represent a vast genetic resource comprising in excess of 80,000 different species. Yeast, fungi and bacteria are also in the direct food chain of fish, crustaceans and mollusks. However, only a very few of these microbes, perhaps less than 10 species, have been exploited for aquaculture feeds. These few species have been used primarily for historical reasons and ease of cultivation. They have not been chosen on the basis of any scientific evidence of superiority as nutritional or therapeutic supplements.

[012] The marine environment is filled with bacteria and viruses that can attack fish and shellfish thereby devastating intensive farms very quickly. Bacteria and viruses can also attack single celled microalgae, so these organisms have evolved biochemical mechanisms to defend themselves from such attacks. Such mechanisms may involve the secretion of compounds that inhibit bacterial growth or viral attachment. Such compounds are called

"prebiotics" and have effects similar to how cranberry juice can prevent bladder infections in humans. When nutritional, therapeutic or protective effects are delivered via the whole live organisms, such as *Lactobacillis* in yogurt, such products are referred to as "probiotics" and the organisms are "probionts". For the purposes of this invention, both types of action will be referred to as probiotic.

[013] Several algal species exhibit antibiotic activity. This activity may be due to certain bioactive constituents in the membranes or cell walls, the protein or the carbohydrate of the positively testing species that inhibit bacterial growth (prebiotics or herein probiotics). Any standard screening technique used to identify antibiotic agents may be used to screen for algae having antibiotic activity, including incubating filter disks soaked in culture broth from the candidate algae on a lawn of the target pathogenic microbe (e.g., Vibrio species). This invention contemplates the use of these "friendly algae" in a probiotic fashion to control the growth of certain "pathogenic microorganisms" in a pond. However, the main aspect of this invention is directed to the use of recombinant microbes or virus infected microbes to deliver the bioactive protein of choice. The recombinant microbes or virus-infected microbes may be tested for antibiotic activity by standard antibiotic screening assays to confirm their activity.

[014] Historically, only bacteria have been used in a probiotic fashion to alter a pond's ecology in order to eliminate or reduce the number of pathogenic bacteria. A problem with the bacterial probiotic approach is that

the existing microbial ecology represents a massive buffer that is difficult to modulate with the introduction of relatively small numbers of alternative bacteria and the results to date have been unimpressive. Furthermore, even if the newly introduced bacteria do bloom, any large increase in bacterial levels in a pond can lower oxygen levels and cause harm to the fish or shrimp. The use of photosynthetic microalgae overcomes this problem as they actually increase oxygen levels. Microalgae have not been considered before as probiotics. Previous experience in the screening of extensive algal culture collections has indicated a number of algal species that exhibit antibacterial or bacteriostatic capabilities. Some of these activities may be anti-Vibrio activity. Such species would be candidates for a high value enrichment feed that delivers both nutritional and antibiotic capabilities. This invention provides an approach to disease control which may be the solution to an impending ecological disaster that will result from the present uncontrolled practice of dumping of toxic chemicals and antibiotics into the water systems to control these bacterial, fungal or viral pathogens.

[015] One of the major disease control problems in shrimp aquaculture today is infection by certain viruses (e.g., White Spot Syndrome Virus and Tara Syndrome Virus). Neither current antibiotic, nor probiotic strategies will work in this situation, and shrimp cannot be vaccinated in a way similar to fish. Shrimp have only a rudimentary immune system so they are particularly susceptible to devastation by viral attacks. This invention provides a solution to this problem using a biological control method using microalgae

as the vector to deliver anti-White Spot antibodies directly to the shrimp. These "Designer feeds" would be a normal part of the diet, but modified to deliver a therapeutic dose of antibody directly to the gut of the shrimp. This approach is known as "passive immunity" because the antibody remains outside the host organism and simply prevents infestation through the gut wall. The invention envisions the use of transgenic algae, yeast, fungi or bacteria to deliver the antibody to the virus. Such probiotics, as envisioned in the invention, do not have to replicate in the target organism for the desired effect to occur. Alternatively, the microbe itself may be infected with a virus that is engineered to produce the antibody of interest. Alternatively, the microbial source may deliver a portion of the virus (e.g. a coat protein or coat proteins) or fragment thereof in order to immunize the shrimp, other shellfish, finfish or other animals.

[016] Antibodies to desired targets, such as White Spot Syndrome Virus or Taura Syndrome Virus, may be prepared by routine immunization and selection of monoclonal antibody producing hybridomas, or by screening viral or bacterial expression libraries of immunoglobulin genes and gene fragments. See "Current Protocols in Immunology," Coligan, et al., eds, Wiley Interscience, 1991, and periodic supplements. Nucleic acid sequences encoding the binding sites of the selected antibodies can be cloned using standard methods (see "Current Protocols in Molecular Biology." Ausubel, et al., eds., Wiley-Interscience, 1987, and periodic supplements), and antibodies may be expressed from recombinant microbes (including algae, see, e.g.,

U.S. Patent No. 6,027,900) or cloned into viruses that infect the desired microbes.

[017] There are a number of bactericidal and bacteriostatic peptides, which will inhibit microbial growth and that include, but are not limited to cecropins, penaeidins, bactenecins, callinectins, myticins, tachyplesins, clavanins, misgurins, pleurocidins, parasins, histones, acidic proteins, and lysozymes. These peptides may be expressed in a microbial biomass such as algae, yeast, fungi or bacteria using recombinant methods as described above, and thus provided as a feed component to convey resistance to infestation.

[018] The invention as contemplated herein, is described in the following examples, but its utility is not limited to the examples provided.

EXAMPLES

[019] **Example 1. Selection of Useful Microbial Sources for Feeds that Provide Disease Control.** Microalgal biomass samples, aqueous extracts, organic extracts and extracts from the growth medium after cultivation of the algae were concentrated and spotted on filter paper discs. Using sterile techniques, these discs were then placed on agar plates overlaid with a lawn of selected organisms including but not limited to gram-negative bacteria, gram-positive bacteria, antibiotic resistant bacteria, yeast, or fungi. After incubation for an appropriate length of time to allow growth of the lawn of test organism the samples were plates were observed for zones of clearing (non-growth) around the filter paper discs. Large zones of clearing indicate

potent antibiotic activity; small zones of clearing indicate less potent antibiotic activity.

[020] **Example 2. Incorporation of an antibody into an algal feed.** A particular viral or bacterial pathogen is chosen and used to prepare monoclonal antibodies using procedures well known to experts in this field. Gene(s) coding for this antibody or an appropriate antibody fragment (Fab or Fv) are isolated and amplified in the appropriate vector. The gene is spliced into a transformation vector suitable for a eukaryotic algae or a prokaryotic alga (e.g. *Synechocystis),* or a yeast (e.g. *Saccharomyces)* or a fungus (e.g. *Mortierella).* The transformation vector is chosen so that the antibody will be over expressed in the microbial cell biomass. This biomass is then used as a feed additive in such a way as to provide the antibody directly to the animal thus providing passive immunity.

[021] **Example 3. Expression of a bactericidal protein in a microbial feed.** A bactericidal protein is chosen for the particular application. For example, proteins of the penaeidin class may be chosen for pathogenic control in shrimp. Penaeidins are members of a family of antimicrobial peptides isolated from crustaceans (e.g., Penaeid shrimp). Antimicrobial peptides may also come from insects and chelicerates and may include but are not limited to cecropins, peneaidins, bactenecins, callinectins, myticins, tachyplesins, clavanins, misgunins, pleurocidins, parasins, histones, acidic proteins, and lysozymes. The gene for the chosen protein or peptide is either isolated from the original source, an amplification source, or it can be made

synthetically. The gene is then incorporated into a transformation vector suitable for a eukaryotic algae (e.g. *Chlorella)* or a prokaryotic alga (e.g. *Synechocystis),* or a yeast (e.g. *Saccharomyces) or* a fungus (e.g. *Mortierella).* The transformation vector is chosen so that the protein will be over expressed in the microbial cell biomass. This biomass is then used as a feed additive in such a way as to provide the bactericidal protein directly to the animal thus providing resistance to that particular pathogen.

[022] **Example 4. Vaccination using Feeds**. An antigen characteristic to a particular pathogen is chosen as is required by the animal and circumstances. For example, a viral coat protein(s) or component thereof, or an infectious bacterial protein, or a component thereof is chosen. The gene coding for the protein(s) is isolated and incorporated into a vector suitable for use in the microorganism of choice. The transformation vector is chosen so that the protein(s) will be over expressed in the microbial cell biomass. This biomass is then used as a feed additive in such a way as to provide the viral or bacterial or fungal protein(s) directly to the animal thus stimulating an immunological response to that particular pathogen. The microbial component may enter the body of the animal in the digestive tract, or otherwise through contact in the air or water.

[023] **Example 5. Vaccination using probiotic Feeds**. Probiotic bacteria such as Lactobacillus, Bacillus, Bifidobacterium, etc. provide beneficial effects by their presence as live organisms in the digestive track of an animal. As such they are constantly replicating and become a significant

portion of the intestinal microflora and make an excellent continuous delivery mechanism for oral vaccines. Oral vaccines must deliver the antigen to a portion of the intestinal mucosa where it can interact with immunogenic tissues (eg., Peyers Patches) and stimulate an immunogenic response. An antigen characteristic to a particular pathogen is chosen as is required by the animal and circumstances. For example, a viral coat protein or component thereof, or an infectious bacterial protein, or a component thereof is chosen. The gene coding for the protein is isolated and incorporated into a vector suitable for use in the probiotic microorganism of choice. Other gut microfloral components not generally considered as probiotics, but which live in the intestine, such as coliforms (e.g. Escherichia coli) can also be used as a vector for producing the vaccine in situ.

[024] The two viral coat proteins from salmon infectious pancreatic necrosis virus (IPNV) are isolated and inserted into a transformation vector selected for use in Lactobacillus using molecular biology methods that are well know in the state of the art. The recombinant Lactobacillus cells expressing the viral antigens as free proteins, excreted proteins, and/or virus like particles (assembled viruses with no nucleic acid) are then grown using conventional fermentation technology, harvested and processed into a form usable as a feed for salmon. This form may include, but is not limited to freeze drying, spray drying, fluid bed drying, microencapsulation, extrusion, or tableting. The recombinant Lactobacillus is then provided to the salmon as a feed, thereby delivering both the valuable probiotic as well as the vaccine. In

this case, the vaccine is constantly produced as long as the recombinant Lactobacillus is present in the gut of the animal.

[025] **Example 6. Delivery of active peptides or proteins using probiotic feeds**. The gene for an active antimicrobial peptide, such as, but not limited to, cecropins, peneaidins, bactenecins, callinectins, myticins, tachyplesins, clavanins, misgurins, pleurocidins, or parasins, or an antimicrobial protein such as histones, acidic proteins, or lysozymes is isolated and inserted into a transformation vector selected for use in *Lactobacillus* using molecular biology methods that are well know in the state of the art. The recombinant *Lactobacillus* cells expressing the free peptides or proteins, or excreted proteins, are then grown using conventional fermentation technology, harvested and processed into a form usable as a feed for an animal such as, but not limited to fish, crustaceans, livestock, etc. This form may include, but is not limited to freeze drying, spray drying, fluid bed drying, microencapsulation, extrusion, or tableting. The recombinant *Lactobacillus* is then provided to the animal as a feed, thereby delivering both the valuable probiotic as well as the antimicrobial compound. In this case, the antimicrobial compound is constantly produced as long as the recombinant *Lactobacillus* is present in the gut of the animal.

WHAT IS CLAIMED IS:

1. An animal feed containing microbial biomass comprising one or more proteins, antibodies, or a combination thereof, wherein said proteins and antibodies are non-native to the microbes of the biomass.

2. The animal feed as claimed in claim 1, wherein the microbes are selected from yeast, fungi, bacteria, algae, or combinations thereof.

3. The animal feed as claimed in claim 1, wherein the microbes are algae.

4. The animal feed as claimed in claim 1, wherein the microbes are yeast.

5. The animal feed as claimed in claim 1, wherein the microbes are bacteria.

6. The animal feed as claimed in claim 1, wherein the microbes are fungi.

7. The animal feed as claimed in claim 1, wherein the microbes express the peptides, proteins or antibodies recombinantly.

8. The animal feed as claimed in claim 1, wherein the microbes are infected with viruses which express the proteins or antibodies recombinantly.

9. The animal feed as claimed in claims 1-8, wherein the feed is used for aquaculture.

10. A method of delivering therapeutic, bioactive or nutritional proteins to an animal comprising administering to an animal a feed comprising a microbe expressing a non-native therapeutic, bioactive or nutritional protein.

11. The method of delivering therapeutic, bioactive or nutritional proteins as claimed in claim 10, wherein the non-human animal is subjected to intensive agricultural practices.

12. The method of delivering therapeutic, bioactive or nutritional proteins as claimed in claim 10, wherein the animal is an aquaculture species.

13. The method of delivering therapeutic, bioactive or nutritional proteins as claimed in claim 10, wherein the microbe is an alga.

14. The method of delivering therapeutic, bioactive or nutritional proteins as claimed in claim 10, wherein the microbe is a fungus.

15. The method of delivering therapeutic, bioactive or nutritional proteins as claimed in claim 10, wherein the microbe is a bacterium.

16. The method of delivering therapeutic, bioactive or nutritional proteins as claimed in claim 10, wherein the microbe is a yeast.

17. The method of delivering therapeutic, bioactive or nutritional proteins or proteins as claimed in claim 10, wherein the therapeutic protein is a recombinant protein expressed by the microbe.

18. The method of delivering therapeutic, bioactive or nutritional proteins as claimed in claim 10, wherein the microbe is infected by a recombinant virus which expresses the therapeutic protein recombinantly.

19. The method of delivering a therapeutic, bioactive or nutritional protein or proteins as claimed in claim 10, wherein the therapeutic protein is a protein which nhibits growth or replication of *Vibrio* species *in vitro*.

20. The method of delivering a therapeutic, bioactive or nutritional protein or proteins as claimed in claim 10, wherein the therapeutic protein is a protein which inhibits a virus.

21. The method of delivering a therapeutic, bioactive or nutritional protein or proteins as claimed in claim 10, wherein the therapeutic protein is a protein which inhibits Taura Syndrome or White Spot Syndrome Virus infection in shrimp.

22. The method of delivering a therapeutic, bioactive or nutritional protein or proteins as claimed in claim 10, wherein the therapeutic protein is a recombinantly expressed antibody.

23. A method as claimed in claims 10-22, wherein the therapeutic, bioactive or nutritional protein is from the following list cecropins, penaeidins, bactenecins, callinectins, myticins, tachyplesins, clavanins, misgurins, pleurocidins, parasins, histones, acidic proteins, and lysozymes.

24. A method as claimed in claims 10-22, wherein the animal is non-human.

25. A probiotic bacteria that carries a vaccine.

26. A method of delivering a vaccine to an animal comprising using a probiotic bacteria expressing that vaccine.

27. A method of delivering a vaccine to an animal comprising using a vaccine-expressing bacteria that grows in the intestine.

28. A method of immunizing an animal by providing to the animal a probiotic bacteria expressing a vaccine in the diet.

29. A probiotic bacteria, which delivers an antimicrobial compound.

30. A method of delivering an antimicrobial compound to an animal comprising using a probiotic bacteria expressing that antimicrobial compound.

31. A method of delivering an antimicrobial compound to an animal comprising using an antimicrobial-expressing bacteria that grows in the intestine.

APPENDIX B

PI 1082309

THE UNITED STATES OF AMERICA

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THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/410,818*
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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

Docket Number 08717.6009		Type a plus sign (+) inside this box →	+

INVENTOR(s)/APPLICANT(s)

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TITLE OF INVENTION (280 characters max)

PROTEIN AND PEPTIDE EXPRESSION FOR PASSIVE IMMUNITY

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification	13 Pages	☐ Small Entity Statement
☐ Drawing(s)	[Number] Sheets [Number] Figures	☐ Other (specify)

METHOD OF PAYMENT (check one)

☒	A check or money order is enclosed to cover the Provisional filing fees	PROVISIONAL FILING FEE
☒	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 06-0916.	☐ $160.00 ☒ $80.00 (small entity)

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE Kenneth J. Meyers Date September 16, 2002

TYPED OR PRINTED NAME Kenneth J. Meyers REGISTRATION NO. 25,146

☐ Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

Attorney Docket No. 08717.6009

PROVISIONAL PATENT APPLICATION

OF

ARUN K. DHAR

AND

F. C. THOMAS ALLNUTT

FOR

PROTEIN AND PEPTIDE EXPRESSION FOR PASSIVE IMMUNITY

BACKGROUND OF THE INVENTION

Passive immunity is the delivery of immune function directly to an animal without the need for an immune response. It is commonly referring to the delivery of antibodies produced in one organism to a naïve organism in order to provide protection from a specific disease or symptom (Zhang et al. 1989; Lorenzen et al. 1990; Lee et al. 1997). An analogous approach is the delivery of a compound or compounds that prevent binding of the infectious agent to its site of infection, either directly or by competition for the binding site. Many drugs are based on this type of interaction.

Viral diseases cause a huge amount of damage in humans, terrestrial and aquatic animals. Organisms that have primitive or poorly developed immune systems are especially susceptible to viral disease. Crustaceans, such as shrimp, do not have adaptive immunity. Instead they rely on the innate immune response. Although several immune genes involved in bacterial and fungal immunity in invertebrates have been well characterized, very few immune genes that are involved in viral pathogenesis are known from shrimp or any other invertebrate, so far.

Viral diseases cause a huge amount of economic loss in crustacean aquaculture. A number of viruses are important to shrimp aquaculture and cause billions of dollars worth of damage annually with virtually no therapeutic treatment available to combat this problem. Yellow head virus (YHV), Taura syndrome virus (TSV), Infectious hypodermal and hematopoietic necrosis virus (IHHNV) and White spot syndrome virus (WSSV) have caused pandemics that have affected global penaeid shrimp farming (Lightner 2002).

White spot disease, caused by the white spot virus (WSV), is currently the most important viral disease of cultured penaeid shrimp (*Penaeus* sp.) worldwide. WSV has a bacilliform enveloped morphology, and the genome contains a circular double stranded DNA of ~292 to 305 Kb (van Hulten et al. 2001; Yang et al. 2001). The SDS-PAGE analysis of purified WSV showed four major polypeptide with estimated molecular mass of 28 kDa (VP 28), 26 kDa (VP 26), 24 kDa (VP 24), and 19 kDa (VP 19) (van Hulten et al. 2000). Out of these four proteins, VP 26 and VP 24 are associated with the nucleocapsids, whereas VP 28 and VP 19 remain with the envelope (van Hulten et al. 2000). Although considerable progress has been made in the detection and molecular characterization of WSV in recent years, efforts to develop therapeutics to prevent white spot disease have not been developed

Molecular methods have been developed to express proteins in yeast (Elledge et al. 1991; McGonigal et al. 1998; Cereghino and Cregg 1999; Cregg et al. 2000), bacteria (Iyer et al. 2002), plants (Mason et al. 1992; Kapusta et al. 1999), fungi (Oyama et al. 2002) and algae (Lapidot et al. 2002; Shapira et al. 2002; Ton et al. 2002). Many of the hosts for the previous systems for protein expression have been used in feeds for terrestrial animals and aquatic animals.

Oral delivery of drugs and vaccines is common (Cho and Howard 1999; Tacket et al. 2000; Bootland et al. 2002). Expression of proteins, including viral proteins, in bacteria, yeast, fungi, plants, animals, and algae as well as tissue cultures thereof is also previously described.

There is a need for new methods to combat viral disease in humans, terrestrial animals and aquatic animals. The need is extreme in cases where the immune system is primitive or poorly developed. One example is crustaceans, where there is a primitive immune system relying not on antibodies but on innate immunity and production of lectins.

SUMMARY OF THE INVENTION

It is an object of the invention to produce a viral protein in yeast, bacteria, plants, fungi, animals, insects, and algae, as well as tissue/cell cultures of these systems, which is orally provided to animals to provide protection from viral infection.

It is an object of the invention to produce a fusion protein containing a viral protein portion that is orally provided to animals to provide protection from viral disease.
It is an object of the invention to protect an animal from viral infection by blocking binding of virus to its receptors in the gut by provision of competing viral protein(s) administered orally.

It is an object of the invention to protect an animal from viral infection by blocking binding of virus to its receptors in the gut by provision of binding moieties of its receptor(s) on the gut.

These and other objects of the invention are provided by one or more of the following embodiments.

In one embodiment of the invention a method of protecting an animal from viral infection is provided. The method comprises the steps of:

Production of a viral protein, such as a capsid or envelope protein, in a foreign host expression system, such as in a yeast, fungus, bacterium, alga, insect, animal, or plant, or alternatively in tissue/cell cultures of these systems.

Processing the biomass containing the viral protein into a feed or feed supplement with minimal purification,

Providing the biomass to the animal to deliver the viral protein in an amount up to 0.5 to 50 % of the total animal feed content,

Wherein the presence of the viral protein competes with live virus inside the animal to prevent infection.

In another embodiment of the invention a method of protecting an animal from a viral infection is provided. The method comprises the steps of:

Production of a receptor or receptor moiety to which a virus attaches for infection in a foreign host expression system, such as in a yeast, fungus, bacterium, algal, insect, animal, or plant or tissue cultures thereof,

Processing the biomass containing the virus-binding receptor into a feed or feed supplement with minimal purification,

Providing the processed biomass to the animal to deliver the virus-binding domain in an amount up to 5% of the total animal feed protein content,

Wherein the presence of the virus-binding domain prevents live virus binding and infection in the animal.

This invention provides a rapid response to a viral disease threat. It can also be applied to other types of diseases caused by bacteria, prions, DNA, protists, and other disease causing organisms or factors.

In organisms without well developed immune systems the invention provides both acute and chronic methods for treatment of disease via delivery of preformed virus proteins, virus binding domains, receptors for the virus, domains from the receptor that bind virus or similar functional units that will tie up either free virus or block binding of the virus to its receptor through competition. These can be delivered chronically or for acute treatment of an infection.

In organisms with well-developed immune systems the invention provides a first response method to retard the onset an acute infection threat until the immune response can be mounted.

The first approach involves expression in a number of different systems (e.g., bacterial, plant, algal, fungal, insect, and yeast and tissue cultures thereof) of a viral protein or proteins. These proteins can be the whole protein or just the domain, which recognizes the virus' receptor on the mucosal lining. These are then fed to the target animal either as whole cells or broken cells or purified or partially purified protein to compete with the virus for binding to the mucosal lining. Such competition will retard or prevent viral infection.

The second approach involves expression in a number of different systems (*e.g.*, bacterial, plant, algal, fungal, insect, and yeast and tissue cultures thereof) of the receptor or binding site of the receptor for the virus of interest. These receptors are limited in only the binding affinity to the virus of interest and can be truncated or modified as needed. The receptor mimetic would bind to the virus to compete with the mucosal receptor and inactivate the virus, thereby preventing infection.

A third approach to preventing the uptake of live WSSV by the shrimp is to provide a high concentration of a viral binding protein in a whole or lysed recombinant cell (or semipurified preparation) such that most, if not all, of the live viral particles will bind to the mimetic and not to the shrimp viral binding site, thereby minimizing infectivity. Dhar and colleagues have studied patterns of differentially expressed genes in shrimp following infection by WSSV (Dhar et al. 2001; Astrofsky et al. 2002) and found a gene coding for a lipopolysaccharide/beta-glucan binding protein (LGBP) to be one of several genes that are up regulated (Roux et al. 2002). Recognition proteins, such as LGBP, play a key role in the NSIR of insects and crustaceans. LGBP may represent the endogenous viral binding protein in shrimp that contains 1,352 base pairs coding for a polypeptide of 376 amino acids in length (Roux et al. 2002). A protein of this size is unlikely to cross the mucosal membrane and therefore may represent the endogenous viral binding protein known to activate the prophenol oxidase cascade. Consequently, if such a protein is delivered in a NC and exposed to the mucosal

tissues, it may specifically bind the virus (similar to an antibody), preventing it from binding to the endogenous binding site in the mucosal tissues and thereby preventing the initiation of the infection process. It may also be desirable to reduce the size of the LGBP by expressing only the LGBP binding domain (*i.e.*, the region that contains the B-1,3-linkage of polysaccharide and the RGD motifs as indicated by consensus sequences with homologous proteins. A truncated protein may have similar binding affinity, but be more resistant to cleavage by endogenous proteinases.

The following examples are provide for exemplification purposes only and are not intended to limit the scope of the invention.

Description & Examples

Definitions

In describing the present invention, the following terminology is used in accordance with the definitions set out below.

"Passive immunity" is defined here as delivery of either antibodies or proteins that deliver protection from infection either by binding to the virus or to its receptor or mode of entry into the animal.

A "primitive immune system" is defined as a system lacking the production of specific antibodies in response to the presence of antigen or having a weak antigen-mediated immune response. This is found in several classes of organisms including but not limited to invertebrates, crustaceans, annelids, nematodes, rotifers, mollusks, echinoderms, insects, chelicerates, protists, ascidians, sponges and corals.

A "target animal" is defined as the animal, which is threatened by a disease-causing element.

A "feed" is defined as a preparation providing nutritional value to any animal, including but not limited to terrestrial animals (humans, cattle, horses, pigs, sheep, goats, poultry) and aquatic animals (fish, shrimp, lobsters, crawfish, mollusks, sponges, jellyfish).

A "feed additive" is anything that is added to an animal's feed, regardless of nutritional value.

EXAMPLES

Example 1. Production of recombinant White Spot Virus proteins VP19 in a yeast expression system.

The gene for WSSV protein VP19 is available from the GenBank database (AF369029). Design primers for the entire VP 19 protein. Perform PCR/ RT-PCR to amplify the entire gene as well as the hydrophilic domains of VP19 gene using standard methods (Sambrook et al. 1989). Cloning of full-length VP19 gene using the pYES2-DES52 *Saccharomyces cerevisiae* expression system (Invitrogen, Inc.) is carried out with GAL1 promoter applied for separate expression of the two viral genes simultaneously under galactose induction. The transformants are screened by

PCR with sequencing of the positive clones to ensure their identity with the original sequence. Western blot detection methods will be used to validate production of protein using standard methods (Sambrook et al. 1989).

Example 2. Production of recombinant White Spot Virus proteins VP28 and VP26 in a yeast expression system.

The genes for WSSV proteins VP 26, and VP 28 DNA are available from the GenBank database (AF173992, AF173993). Design primers for the entire VP 26 and VP 28 proteins. Perform PCR/ RT-PCR to amplify the entire gene as well as the hydrophilic domains of VP 26 and VP 28 genes using standard methods (Sambrook et al. 1989). Cloning of full-length VP26 and VP 28 genes using *Saccharomyces cerevisiae* expression system pESC (Stratagene) is carried out with Gal1 and Gal10 promoters applied for separate expression of the two viral genes simultaneously under galactose induction. The transformants are screened by PCR with sequencing of the positive clones to ensure their identity with the original sequence. Western blot detection methods will be used to validate production of protein using standard methods (Sambrook et al. 1989).

Example 3. Method for protection of shrimp from WSSV infection.

Shrimp are fed recombinant *Saccharomyces cerevisiae* containing proteins derived from WSV coat protein genes (as in Examples 1 and 2), these proteins appear to block the viral receptors needed for WSV infection to provide a passive immunity to the animals and provide some protection from WSSV disease. The yeast are provided in either whole or broken form directly to the fish in a microbound format in beads composed of alginate and starch in a polymeric form. Alternative microbound forms are available such as polyactide (Bootland et al. 2002), carrageen, alginate, and chiotsan. Attractants can be added to make the beads more easily consumed by the target species (in the case of shrimp, krill meal would be a good alternative). A challenge with the WSSV will result in increased survivability in response to viral infection in shrimp fed the recombinant yeast.

Example 4. Production truncated recombinant White Spot Virus proteins VP28 and VP26 in a yeast expression system

The genes for WSSV proteins VP 26, and VP 28 DNA are available from the GenBank database (AF173992, AF173993) as in Example 2. Using the hydrophilicity profile of VP 26 and VP 28 proteins the hydrophilic domains are identified. Perform PCR/ RT-PCR to clone the truncated VP26 and VP 28 using the pESC *Saccharomyces cerevisiae* expression system (Stratgene) using standard methods (Sambrook et al. 1989). This is followed by screening of recombinant clones by PCR and sequencing the clones to ensure their identity with the original sequence. Assay of recombinant protein production by Western blot analysis using WSV VP 26 and VP 28 antibodies. Antibodies are available for VP26 (DiagXotics, Inc., CT) and are made as polyclonals by custom contract with Immuno-Precise Antibodies (Victoria, Canada).

Example 5. Production of LGBP in yeast expression system.

Dhar and colleagues have cloned and sequenced shrimp LGBP gene and the nucleotide sequence is available in the GenBank database (AF473579) (Roux et al. 2002). LGBP is a known elicitor of prophenoloxidase (ProPO) cascade in arthropods. The ProPO cascade is one of the well-characterized defense mechanisms of invertebrates. The entire LGBP gene encoding an open reading frame of 326 amino acid will be cloned in a yeast expression system (pYES2.1 TOPO TA expression system, Invitrogen Inc.). The truncated LGBP gene that contain the putative binding site for β-1,3 linkage for polysaccharide and the cell attachment binding domain (RGD motif) will also be amplified by RT-PCR and cloned into yeast expression cassett (pYES2.1 TOPO TA expression system, Invitrogen Inc.). Recombinant protein is made out of the binding region and expressed in yeast expression system as in Example 1.

Example 6. Protection of shrimp from WSSV infection using recombinant LGBP of Example 5.

Yeast from Example 6 are mixed with the feed either in a microbound format (as in Example 3) or directly in cold extruded feeds. Feeds are then provided to shrimp and protection is provided from infection by WSSV by the binding of LGBP to WSSV and the activation of the ProPO cascade.

Example 7. Production of recombinant IPNV VP2 protein in bacteria.

Genes for the VP2 capsid protein of infectious pancreatic necrosis virus, a fish virus, are cloned according to existing literature (Yao and Vakharia 1998). The gene is cloned into pTrcHis vector (Invitrogen), a protein expression vector for *Escherichia coli*. The protein is expressed behind the *Trc* promoter (a version of the *Trp* promoter) and expressed in the cell. Whole cells are harvested that contain the gene on induction by either IPTG (isopropyl-1-β-D-galactoside) or other inducer of the *Trc* promoter. Production of the recombinant protein is validated by western analysis using standard methods and antibody for Immuno-Precise Antibodies produced to IPNV isolated at our laboratory (Sambrook et al. 1989).

Example 8. Protection of fish from IPNV infection using recombinant IPNV VP2 protein expressed in bacteria.

Recombinant bacteria from Example 7 are fed, either formulated, encapsulated or directly, to fish (such as hybrid striped bass or salmon) at a final recombinant protein concentration of less than 100 mg/kg. The VP2 competes with virus for binding to the receptors within the gut of the fish to provide protection from IPNV infection.

Example 9. Protection of shrimp from WSSV infection by expression of ligand binding domain of the virus.

Recombinant green algae (*Chlorella vulgaris*) are produced using established methods that are expressing the receptor for WSSV (Choi et al. 2000). The cells are grown either photosynthetically in enclosed photobioreactors (Rebolloso-Fuentes et al. 2001; Lebeau et al. 2002) or in traditional fermentors (Running et al. 1994).

Recombinant cells are fed directly to shrimp experiencing an outbreak of WSSV to prevent binding of the virus to its receptor, thereby preventing disease.

Example 10. Acute protection of animals that have an highly developed immune system.

Animals such as humans, terrestrial agricultural animals (*e.g.*, cows, horses, sheep, swine, rabbits, goats), aquatic animals (*e.g.*, fish) and pets (*e.g.*, dogs, cats) that have a higher immune system can be protected in a manner analogous to that for the primitive immune system animals described in Example 9 or Examples 1-3 as protection from initial infection during an outbreak prior to the induction of antibody production. Virus receptors or viral proteins that mediate binding the receptor can be provided as a first response to an infection to protect the animal while the body begins to respond with the immune system.

Example 11. Expression of WSSV proteins in a green alga, *Chlorella vulgaris*.

The genes coding for VP19 and VP28 are ligated into the *pCNR/HUP* vector at a site downstream and under control of the *NR*-promoter (nitrate reductase) to generate the transformation plasmids *pCNR/HUP/VP19 and pCNR/HUP/VP28*. These plasmids will be used to transform $HUP^{(-)}$ *Chlorella* (hexose uptake minus mutants) using the particle bombardment procedure (Biolistics®) and transformants will be selected by growth in the dark on glucose (Allnutt et al. 2000). Transformed colonies will be subcultured and tested for the production of the presence of VP19 and VP28 by Western blot analysis using antibodies using standard techniques (Sambrook et al. 1989).

The binding affinities of VP19 and VP28 relative to intact WSSV will be determined using a standard competitive binding assay (Chan and Perlstein 1987) and anti-WSSV antibody coated microplates. WSSV labeled with a fluorescent marker (*e.g.*, phycocyanin) will be added to each well of the microplate along with serial dilutions of the extracts from the VP19 or VP28 producing NC's. The titration curve of the fluorescence provides an estimate of the binding affinity of the viral mimetic relative to the virus itself. Truncated versions of the virus proteins can also be made that deliver similar binding affinities to be used for provision of passive protection against WSSV infection.

The recombinant *C. vulgaris* cells expressing the WSSV virus proteins can be fed directly to shrimp or supplied as component in the feeds as described in Examples 3 and 6.

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We claim:

1. A method of protecting an animal from disease comprising steps of:
introduction of a disease related protein into a cell, said protein being responsible for targeting the disease-causing element to the gut,
production of the protein in the transformed cell,
delivery of the protein from the transformed cell to the target animal,
wherein the binding of the disease-causing element is inhibited or retarded.

2. The method of Claim 1, wherein the disease-causing element is a virus.

3. The method of Claim 1, wherein the cell transformed is chosen from the following possible sources, bacterial, algal, yeast, fungal, insect, animal, and plant or tissue cultures of any of this list.

4. The method in Claim 1, wherein the disease-causing element is a bacteria.

5. The method in Claim 2, wherein the disease-causing element is a prion.

6. The method of Claim 1, wherein the cell transformed is an alga.

7. The method of Claim 1, wherein the cell transformed is a yeast.

8. The method of Claim 1, wherein the cell transformed is a bacterium.

9. The method of Claim 1, wherein the protein from the disease-causing element is from a virus.

10. The method of Claim 1, wherein the expressed protein comprises a segment from white spot syndrome virus.

11. The method of Claim 10, wherein the expressed protein comprises a segment from white spot syndrome virus protein VP26.

12. The method of Claim 10, wherein the expressed protein comprises a segment of white spot syndrome virus protein VP28.

13. The method of Claim 10, wherein the expressed protein comprises a segment of white spot syndrome virus protein VP19.

14. The method of Claim 10, wherein the expressed protein comprises a segment of white spot syndrome virus protein VP24.

15. A feed that is supplemented with a recombinant protein or peptide that competes with a disease-causing element to reduce or alievate a disease state.

16. A feed as in Claim 15 that is supplemented with a recombinant protein or peptide comprising viral sequences.

17. A feed as in Claim 15 that is supplemented with a recombinant protein or peptide comprising white spot syndrome virus sequences.

18. A feed as in Claim 17 that is supplemented with a recombinant protein or peptide that comprises sequences of white spot syndrome virus proteins from one or more of the following proteins, VP24, VP28, VP26 and VP19.

19. A feed additive, either fed as whole cells, broken cells, semi-purified protein, purified protein or encapsulated versions of these forms, that contains a recombinant protein or peptide that competes with a disease-causing element to reduce or alievate a disease state.

20. A feed additive as in Claim 19 that is supplemented with a recombinant protein or peptide comprising viral sequences.

21. A feed additive as in Claim 19 that is supplemented with a recombinant protein or peptide comprising white spot syndrome virus sequences.

22. A feed additive as in Claim 21 that is supplemented with a recombinant protein or peptide that comprises sequences of white spot syndrome virus proteins from one or more of the following proteins, VP24, VP28, VP26 and VP19.

23. A method of protecting an animal from disease comprising steps of:

introduction of a target animal protein moiety into a cell, said protein moiety being capable of binding to a disease-causing element,

production of the target animal protein moiety in the transformed cell,

delivery of the target animal protein moiety from the transformed cell to the target animal,

wherein the binding of the disease-causing element is inhibited or retarded.

24. A feed that is supplemented with a recombinant protein or peptide that protein moiety being capable of binding to a disease-causing element to reduce or alievate a disease state.

25. A feed additive, either fed as whole cells, broken cells, semi-purified protein, purified protein or encapsulated versions of these forms, protein moiety being capable of binding to a disease-causing element.

ABSTRACT

This invention relates to feeds, feed supplements and methods for their use that provide disease controlling properties to humans, terrestrial and aquatic animals. These methods and compositions have both acute and chronic applications. Chronic applications are especially critical to the health of organisms that have primitive or poorly developed immune systems.